AGE FACTOR IN THE MATURATION OF COLLAGEN. CROSS-LINKS IN HEAT-DENATURED COLLAGEN IN TAIL TENDON AND SKIN OF RAT

FORMATION of intermolecular cross-links at aging of the organism has been repeatedly suggested (Verzár, 1958, 1963; Gross, 1961). Direct evidence is scarce, because of technical difficulties in estimating the cross-links, especially between globular protein molecules, but the hypothesis can be tested with fibrous protein, e.g. with collagen. The study of cross-links is also of intrinsic significance for understanding of the mechanical properties of collagenous fibres and their relation to chemical linkages between the peptide chains and individual tropocollagen molecules.

Wiederhorn and collaborators (Wiederhorn and Reardon, 1952; Wiederhorn et al., 1953) have developed a method for the estimation of the cross-links of denatured collagen which is based on the statistical elasticity theory of Flory and Rehner (1943). The method has since been used, among others, in modified form by Cater (1963) in the study on the effect of various in vitro-treatments on collagenous fibres. The purpose of the present work was to apply the method to the study of the rat skin and tail tendon collagen with emphasis on the age of the animals. A preliminary report has appeared (Kulonen et al., 1963).

EXPERIMENTAL

Theory. The basic relationship is as follows:

$$f = \frac{RT\rho V_2^{\frac{1}{3}}}{M_c}(\alpha - 1/\alpha^2)$$

f = force in g/cm^2 of the cross-sectional area of the non-extended fibre,
R = gas constant, $8\cdot478 \times 10^4$,
T = absolute temperature,
V_2 = volume fraction of dry sample from the swollen sample,
ρ = density of the dry material,
α = ratio of the extended fibre to non-extended fibre,
M_c = average molecular weight between the junction points.
The value of V_2 was calculated from the formula

$$V_2 = \frac{W_0 \times d}{W \times \rho - W_0(\rho - d)}$$

W_0 = weight of the dry sample,
W = weight of the swollen sample,
ρ = density of collagen, accepted as $1\cdot3$,
d = density of the imbibing fluid.

Material. The ages of the Wistar rats were known to an accuracy of a few days. The rats had never been used in any experiments. After killing the skins and tails had been stored at −18°C.

The tail tendon fibres (length 10–15 cm) were extracted from the tips of the tails with forceps and immediately denatured for 30 sec in water at 70°C. The process of denaturation was ended by immersion of the fibres into cold water. The samples were air-dried at room temperature.

The skins were cut to strips, 10 cm long and 2–3 mm broad. The globular proteins were removed by extraction with 10% (w/v) sodium chloride solution for 15 hr under shaking. The fats were removed by extraction with mixture of ethanol and ethylether (3:1, v/v) for 5 hr. Suitable denaturation (cf. Discussion) was obtained in 90 sec in water at 70°C. The samples were then immersed in cold water and air-dried at room temperature.

Before stress–strain analyses the samples were kept in water at 40°C for at least 2 hr, where the experiment was carried out. The length of the swelling period had been found by preliminary experiments.

Measurement of the stress–strain relationship. The temperature in the water bath was 40.0 ± 0.1°C. The sample (length about 4 cm, measured exactly with a kathetometer) was clamped between the torsion balance (Vereenigde Draadfabrieken, division 0·005 g) and a specially constructed frame (Fig. 1). The lower clamp could be moved vertically with an accuracy of 0·1 mm. The sample was extended at 1·0 mm steps and the tension
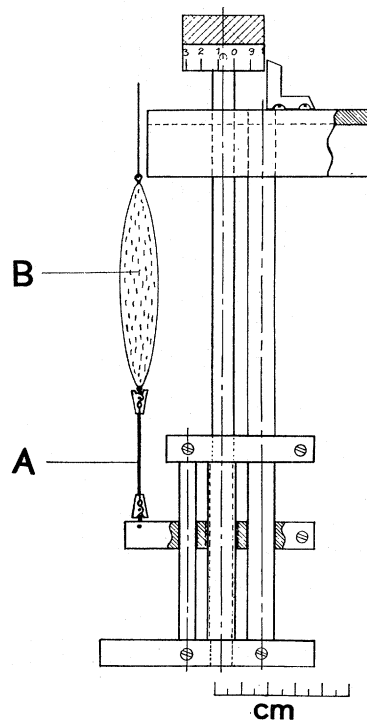
was read from the balance after 3 min equilibration time to an accuracy of 5 mg. If the extension exceeded about 5 mm, the increment in the stress was no longer linear, but larger. A difficulty was encountered in the measurement of non-extended length, since some initial elongation was due to the straightening of the sample. Therefore those M_c values, which are calculated from later extension points, show less dispersion and are thus considered more reliable. Finally, the sample was allowed to retract to its original length by similar steps.

Additional measurements. The average cross-sectional areas were calculated from the length and volume of the samples. Unsuccessful attempts were made by visual estimation of the thickness of the tail tendon fibres with the microscope, but the accuracy was not satisfactory. After stress–strain analysis the sample was cut from the clamps, dried swiftly with blotting paper and weighed both in air and in water on another torsion balance to an accuracy of 0·01 mg. The volume was calculated from these data. The fibres of tail tendons weighed in air 3–15 mg and in water 0·20–0·90 mg, the skin strips in air 130–230 mg and in water 1·5–8 mg.

The dry weight for the calculation of the volume fraction was obtained after 15 hr dehydration of the samples in acetone and subsequent drying for 1 hr at 70°C.

Comments. The most important sources of error are the measurement of the cross-sectional area and the determination of non-extended length. The calculated value of M_c of a sample at various steps of extension was remarkably constant. As an example may be mentioned a series of measurements on tail tendon fibre of 3·69 cm length (wet weight 8·0 mg, weight in water 0·57 mg and in dry state 1·59 mg). The following data were obtained:

extension 1 mm, force 25·0 g/cm², M_c 55·000
extension 2 mm, force 50·0 g/cm², M_c 55·600
extension 3 mm, force 75·0 g/cm², M_c 54·800
extension 4 mm, force 102·5 g/cm², M_c 53·600
extension 5 mm, force 127·5 g/cm², M_c 51·400

RESULTS

Rat tail tendon. It was not possible to study rat tail tendon fibres from rats younger than 3 months, because the samples were fragile and tended to dissolve at denaturation. The data on 3–24 month old rats are shown in Table 1. There were no significant differences in the M_c-values between the age groups, which is confirmed by the constancy of the slope $1/M_c$ as shown in Fig. 2. The normal range of the values of different samples

TABLE 1. THE AVERAGE MOLECULAR WEIGHT BETWEEN THE JUNCTION POINTS (M_c) IN HEATED TAIL TENDON COLLAGEN OF RATS OF VARIOUS AGES

Standard deviation of M_c is indicated, as also the number of fibres (n)

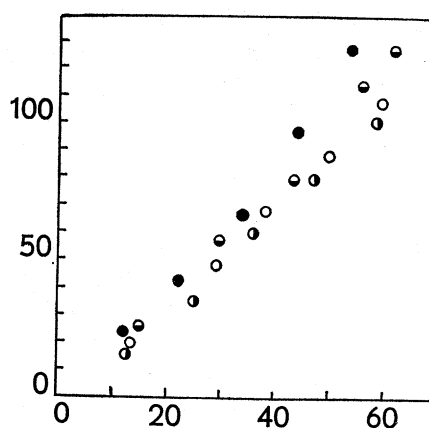| Age of rats	Average M_c
3 months (2 rats)	50,000 ($n = 2$)
6 months (3 rats)	55,500 ± 8900 ($n = 10$)
12 months (3 rats)	67,500 ± 9900 ($n = 8$)
24 months (3 rats)	50,400 ± 10,000 ($n = 8$)

FIG. 2. The stress–strain plot of tail tendon fibres of rats of 3–24 months. The slope of the regression curve is $1/M_C$. Ordinate: force g/cm^2; abscissa: $\rho RTV_2^{\frac{1}{3}}(\alpha - 1/\alpha^2) \times 10^{-5}$. The different symbols indicate the ages of the rats: ● 3 months, ◒ 6 months, ◑ 12 months and ○ 24 months.
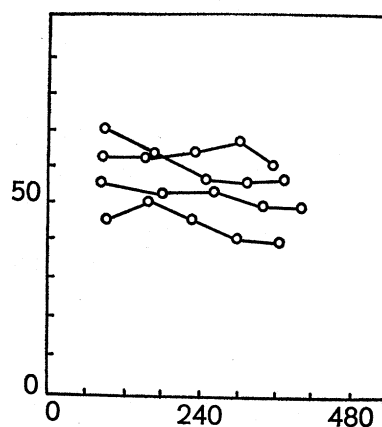

FIG. 3. M_c (in thousands) of rat tail tendon collagen plotted against the extension function $(\alpha - 1/\alpha^2) \times 10^3$. The figure shows the relative constancy of the calculated M_c at various steps of extension and also the ranges between various fibres from the tail tendons of two rats of 6 months.

TABLE 2. THE AVERAGE MOLECULAR WEIGHT BETWEEN THE JUNCTION POINTS (M_c) IN HEATED SKIN COLLAGEN OF RATS OF VARIOUS AGES

Age of rats	Average M_c	Range*
3 months	213,000	168,000–301,000
6 months	99,500	96,100–103,200
12 months	49,800	37,500–63,900
24 months	40,000	26,400–51,000

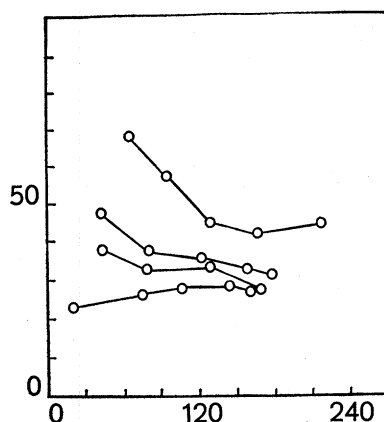* Five samples in each age group, derived from two rats.

FIG. 4. M_c (in thousands) of rat skin collagen plotted against the extension function $(\alpha - 1/\alpha^2) \times 10^3$ analogously with Fig. 3. At small extensions the values do not seem reliable.

at different extensions is shown in Fig. 3. Fibres contain usually weaker sections, which contribute to the dispersion of the results.

Rat skin. The analysis was more complicated than with the tendon fibres and especially the M_c-values, calculated at the early points of extension, had a large dispersion (Fig. 4). From Table 2 it is evident, however, that the age has a very distinct effect on the stress–strain relationship, which is reflected in the continuous decrease of M_c with advancing age and a consequent increase of the number of cross-links.

DISCUSSION

The difference between the skin and tendon is evidence for different structure or, at least, for different formation of cross-links. There are several possibilities for the structure of cross-linking: bridges directly between the peptide chains, linkages with additional carbohydrate or even by non-collagenous proteins (Partington and Wood, 1963). In the preliminary experiments we found that the absolute value of M_c depended on the length and temperature of denaturation. Therefore, we suggest that there are cross-links of varying strength (cf. Verzár, 1964). The presented M_c-values are to be taken only as relative values, which are always larger than those in the original collagen.

The intramolecular cross-links may contribute to the final results. If there are non-linked peptide chains inside the mesh-work of denatured collagen, they contribute to the cross-sectional area, but not to force, and therefore too high M_c is obtained.

Preliminary experiments were made in an attempt to influence the cross-linking biologically *in vitro*. When tendon fibres were incubated with granulation tissue homogenate, the effect, if any, was to increase the M_c or to break the cross-links. Of the production of inter- and intramolecular cross-links in collagen in the living organism we know almost nothing. There is no information on the relationship *in vivo* between tensile strength and amount of cross-linking. It is also a matter of speculation, whether similar cross-links exist between other proteins.

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Summary—The statistical stress–strain theory of elasticity was applied to heated rat tail tendon fibres and skin strips to estimate the amount of cross-links in collagen. In the tail tendon collagen the average molecular weight between the junction points (M_c) did not change from about 50,000, which was observed at 3 months of age. In skin strips the M_c decreased with advancing age from about 210,000 in rats of 3 months to 40,000 in rats of 24 months.

Résumé—La théorie des tensions et allongements statistiques de l'élasticité a été appliquée à des fibres tendineuses et lambeaux cutanés chauffés de la queue du rat afin d'établir le nombre de chaînons du collagène. Dans le collagène du tendon de la queue le poids moléculaire moyen entre les points de jonction (M_c) n'a pas présenté de variations avec l'âge en partant du chiffre de 50.000 observé à l'âge de 3 mois. Dans les lambeaux cutanés le M_c a baissé progressivement en fonction de l'âge, allant de 210.000 chez des rats de 3 mois pour n'atteindre que 40.000 chez des rats de 24 mois.

Zusammenfassung—Zur Bestimmung der Anzahl der Querverbindungen in Kollagen wurden erhitzte Sehnenfasern und Hautstreifen von Rattenschwänzen nach der statistischen Elastizitätstheorie einem Spannungs-Dehnungs-Zustand unterworfen. Bei dem Kollagen der Schwanzsehnen änderte sich das durchschnittliche Molekulargewicht zwischen Verbindungspunkten (M_c) nicht gegenüber dem von ungefähr 50.000, das bei drei Monate alten Ratten festgestellt wurde. In den Hautstreifen sank das M_c mit zunehmendem Alter von 210.000 bei drei Monate alten Ratten auf 40.000 bei 24 Monate alten Ratten.

Резюме — Статистическая теория упругости и зависимости деформации от напряжения применялась к нагретым волокнам сухожилий из хвостов крыс и к полоскам кожи, с целью выяснения количества поперечных связей в коллагене. В коллагене сухожилий хвоста средний молекулярный вес между соединительными пунктами (M_c) не был изменен с, приблизительно, 50,000, цифры, отмеченной для возраста в 3 месяца. В коже (M_c) уменьшилось с возрастом от, приблизительно, 210,000 у крыс возрастом в 3 месяца, до 40,000 у крыс возрастом в 24 месяца.